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Running title: Arsenic alters stromal tumor microenvironment

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ABSTRACT

Background: The tumor microenvironment plays in an important role in the progression of cancer by mediating stromal-epithelial paracrine signaling, which can aberrantly modulate cellular proliferation and tumorigenesis. Exposure to environmental toxicants, such as inorganic arsenic (iAs), has also been implicated in the progression of prostate cancer.

Objective: The role of iAs exposure on stromal signaling in the tumor microenvironment largely has been unexplored. Our objective was to elucidate molecular mechanisms of iAs-induced changes to stromal signaling by an enriched prostate tumor microenvironment cell population, adipose-derived mesenchymal stem/stromal cells (ASC).

Results: ASC conditioned media (CM) collected after one week of iAs exposure increased prostate cancer cell viability compared to ASC CM which received no iAs exposure. Cytokine array analysis suggested changes to cytokine signaling associated with iAs exposure. Subsequent proteomic analysis suggested a concentration-dependent alteration to the HMOX1/THBS1/TGFβ signaling pathway by iAs. These results were validated by quantitative RT-PCR and Western blot, confirming a concentration-dependent increase in HMOX1 and decrease in THBS1 expression in ASC following iAs exposure. Subsequently, utilizing a TGFβ pathway reporter construct we confirmed a decrease in stromal TGFβ signaling in ASC following iAs exposure.

Conclusions: Our results suggest a concentration-dependent alteration of stromal signaling, specifically attenuation of stromal-mediated TGFβ signaling following exposure to iAs. Our results indicate iAs may enhance prostate cancer cell viability through a previously unreported stromal-based mechanism. These findings illustrate that the stroma may mediate the effects of iAs in tumor progression, which may have future therapeutic implications.

INTRODUCTION

Inorganic arsenic (iAs) is a ubiquitously distributed environmental toxicant, classified as a Group 1 carcinogen by the International Agency for Research on Cancer (IARC 2011). The deleterious effects of iAs exposure on human health have been observed for thousands of years (Bolt 2012) and continue to be a major concern today. Recent evidence estimates that nearly 137 million people worldwide are exposed to levels of iAs in their drinking water that exceed the recommended safety limits of 10 ppb mandated by the U.S. Environmental Protection Agency (EPA) (EPA 2001; Ravenscroft 2007).

Both epidemiological and experimental results have suggested a direct link between iAs exposure and prostate cancer (García-Esquinas et al. 2013; Lewis et al. 1999; Prins 2008; Tokar et al. 2010; Treas et al. 2013). The most prevalent and second deadliest form of cancer in men (ACS 2015), prostate cancer costs the U.S. health care system nearly 10 billion dollars annually (Roehrborn and Black 2011). Unfortunately, the propensity for prostate cancer to become resistant to conventional chemotherapeutics by losing sensitivity to androgen ablation therapy makes it very difficult to treat (Feldman and Feldman 2001). This is partially due to the fact that prostate cancer often produces secondary bone metastases, which are associated with poor survival (Ye et al. 2007). Thus, determining whether environmental exposures play a role in the etiology of prostate cancer may reduce the associated health burden by preventing malignant tumor progression.

While the causal relationship between iAs exposure and cancer is well established (IARC 2011), the precise mechanism(s) underlying iAs-induced carcinogenesis have yet to be fully characterized. Several studies have suggested production of reactive oxygen species (Pi et al. 2002), genome instability (Sciandrello et al. 2004), and aberrant expression of DNA repair

machinery (Martinez et al. 2011) as potential mechanisms. However, many of these epithelial-centric hypotheses have not explored the potential contribution of stromal cells within the tumor microenvironment in mediating the tumorigenic effects of iAs.

The tumor microenvironment consists of a variety of biologically active stromal cells, which dynamically secrete a large spectrum of cytokines and growth factors (Witz 2008). Many of these released paracrine factors have been associated with the progression of prostate cancer (Chung et al. 2005). The prostate possesses a unique microenvironment that includes the periprostatic adipose tissue layer, whose increased thickness has been tied to prostate cancer severity and poor prognosis (Finley et al. 2009; Ribeiro et al. 2012b; van Roermund et al. 2011). One specific cell population that is enriched in the prostate microenvironment are the adipose-derived mesenchymal/stromal stem cells (ASC) (Ribeiro et al. 2012c).

Several reports have established that mesenchymal stem cells (MSC), including ASC, possess a tropism towards areas of increased inflammation such as tumors (Spaeth et al. 2008; Zolochevska et al. 2012). It is thought that these cell types are recruited into tumors to promote healing and potentially quell the inflammatory response (Aggarwal and Pittenger 2005; González et al. 2009). While many investigators, including us, are utilizing ASC as a potential therapeutic modality due to their inherent tropism towards tumors (Hall et al. 2007; Zolochevska et al. 2014), emerging evidence has suggested that endogenous ASC also may play a critical role in tumor progression, depending on the cellular context (Barron and Rowley 2012; Kidd et al. 2012). Moreover, the potential effects of environmental toxicants such as iAs on these endogenous cells is unknown. We hypothesized that iAs exposure of a stromal cell population enriched in prostate tumors (ASC) would modify their cell-cell communication with epithelial tumor cells. Therefore, in our experimental design, our goal was to establish (1) whether changes

in cell-cell communication between ASC and prostate cancer cells following exposure to iAs can be detected, (2) whether potential mechanisms for the iAs-mediated ASC alterations can be identified, and (3) whether *in vitro* data can be used to assess how iAs-mediated changes in ASC might enhance prostate cancer progression.

To the best of our knowledge, our results indicate for the first time that exposure to iAs alters stromal-epithelial signaling by modulating global cytokine signaling in a population enriched in the prostate tumor microenvironment (ASC), which may explain the enhanced effects on prostate cancer cell viability. Specifically, we have identified that iAs exposure in ASC aberrantly modulates TGFβ signaling, a pathway strongly linked with the regulation of prostate cancer progression (Li et al. 2008; Santamaria-Martínez et al. 2009). We propose that potential rewiring or reprogramming of stromal cells within a tumor can culminate in an environment which is more suitable for the progression of prostate cancer, thus identifying a novel mechanism in which iAs exposure may elicit its carcinogenic effects.

MATERIALS AND METHODS

Cell Culture and iAs Exposure. The human prostate cancer cell line PC3 was obtained from American Type Culture Collection and maintained in 10% Fetal Bovine Serum (FBS, Fisher) Roswell Park Memorial Institute medium 1640 (RPMI, Corning). ASC from two male Caucasian donors, generously provided by Dr. Jeffrey Gimble, were collected as previously described (Zolochevska et al. 2014) and cultured on fibronectin-coated plasticware in modified media conditions described previously (Ruiz et al. 2010). ASC from Donor 1 were isolated from midsection liposuction, while Donor 2 ASC were isolated from liposuction samples from breast tissue. Briefly, "ASC media" was made to a final concentration of 60% Dulbecco's Modified Eagle Medium (DMEM, Corning), 40% MCDB-201 medium (Sigma), 5% FBS, 1X insulin-

transferrin-selenium (Corning), 1 nM dexamethasone (Acros Organics), 10 ng/mL epidermal growth factor (eBioscience), 0.1 μM ascorbic acid (Acros Organics), and 1X antibiotic-antimycotic (Gibco). For iAs exposure, culture media with either 0, 1, 10, 75 ppb iAs (sodium arsenite, RICCA) was used. All ASC experiments used cells with a passage number <10.

iAs Cytotoxicity. 10^3 ASC were seeded per well in a 96-well plate format in 100 μl ASC media. Baseline cell viability was determined after 24 hr by adding 10 μL of CCK-8 (Cell Counting Kit-8, Dojindo), and incubated for 2 hrs at 37°C. CCK-8 is a water soluble tetrazolium salt that upon entry into a live cell is rapidly reduced by intracellular dehydrogenases forming a spectroscopically active formazan compound, which is proportional to the number of live cells present (Ishiyama et al. 1997). Absorbance was read at 450 nm using a Glomax plate reader (Promega). Media was replaced with ASC media containing varying amounts of iAs and reassessed by CCK-8 assay after 48 hrs.

ASC Cell Cultured Conditioned Media (CM) and PC3 Co-Culture. ASC were exposed to 0 or 75 ppb iAs ASC media for one week as described above. ASC were washed to remove residual iAs and media was exchanged to a low serum media 2% FBS DMEM:F12 (Corning), and incubated without the presence of iAs for an additional 24 hours. CM was collected and stored at -80°C. PC3 cells were seeded at a density of $2x10^3$ cells/well in a 96-well plate format and allowed to adhere for 24 hours. Cell viability was determined (CCK-8) and media was replaced with 2% FBS DMEM:F12 (control), or 2% FBS DMEM:F12 containing CM from ASC previously exposed to 0 or 75 ppb of iAs reflecting a 1% ASC proportion relative to PC3 cells. Cell viability was reassessed by CCK-8 following 48 and 96 hours.

Global Cytokine Expression Array. Briefly, ASC exposed to one week of 0, 1, 10, or 75 ppb iAs were collected. Results were analyzed using Bio-Plex Pro™ Human Cytokine 27-plex

Assay (Bio-Rad) according to the manufacturer's instructions. Cytokine concentrations were determined using Bio-Plex system.

Nontargeted Proteomic Analysis and Bioinformatics. Sample preparation and nanoLC-MS/MS analysis for nontargeted proteomics was performed as previously described (Lichti et al. 2014), with minor modifications. Data files were analyzed as previously described (Lichti et al. 2014). Briefly, automatic and manual alignment of *m/z* and retention time was performed using Progenesis QI for Proteomics (version 18.214.1528, Nonlinear Dynamics), and the top 5 spectra for each feature were exported for database searching in PEAKS (Han et al. 2005; Ma et al. 2003; Zhang et al. 2012) (version 6, Bioinformatics Solutions Inc.) and Mascot (version 2.3.02, Matrix Science) against the UniprotKB/SwissProt Human database (June 2014 version, 20,213 proteins) appended with the Common Repository of Adventitious Proteins contaminant database (2012.01.01 version, http://www.thegpm.org/crap/). After importing the resulting peptide-spectrum matches (95% peptide probability) into Progenesis QI, normalized peptide intensity data for unique peptides were exported and filtered to remove methionine-containing peptides (Perrin et al. 2013) and all modified peptides except cysteine carbamidomethylation. Peptide intensities were imported into DanteR (version 0.1.1) (Karpievitch et al. 2009; Polpitiya et al. 2008) and processed for protein quantification as previously described (Lichti et al. 2014). The resulting proteins were assessed for statistical significance between groups by a two-way ANOVA, with ASC donor and iAs dosage as factors, and *p*-values were corrected for multiple testing (Benjamini and Hochberg 1995).

Ingenuity Pathway Analysis (IPA). IPA (Ingenuity Systems, version 18488943, build 308606M, created June 23, 2014, www.ingenuity.com) was used to analyze proteomics datasets containing protein identifiers, fold-changes, and *p*-values from 2-way ANOVA analyses

comparing ASC exposed to either 0 or 75 ppb iAs for one week in both donors. Predicted network analysis was performed using proteins with a *p*-value <0.05.

qRT-PCR. Total RNA was isolated using the RNeasy Mini Kit (Qiagen). 1 μg RNA was reverse-transcribed using *amfiRivert* Platinum cDNA Synthesis Master Mix (GenDEPOT). Real-time qPCR reactions contained 1 μL cDNA template, 2X SYBR Green Master Mix (Applied Biosystems), and 10 mM forward and reverse primers for both experimental and β-actin controls. qRT-PCR was performed on an Eppendorf Realplex 2S (Eppendorf), using: 40X 95^{o}C for 3 min; 95^{o}C for 3s; 60^{o}C 30s; 72^{o}C 8s and analyzed using EP Realplex software (version 2.2).

Western Blot Analysis. Total proteins were isolated using RIPA Buffer (Thermo Scientific) and freeze-thawing at -80^{o}C in the presence of 1X Halt Protease Inhibitor (Thermo Scientific), 5 mM EDTA, and Phosphatase Inhibitor Cocktail 2 and 3 (Sigma-Aldrich). Protein concentration was determined using BCA Protein Assay (Thermo Scientific) and 50 μg protein was separated via electrophoresis using Bolt 4-12% Bis-Tris Plus Gel (Life Technologies) and transferred onto membranes using the Iblot2 (Life Technologies) system, according to the manufacturer's instructions. Immunoblotting was performed using antibodies for Heme Oxygenase-1 (HMOX1) (#13248, Abcam; 1:250) and Thrombospondin-1 (THBS1) (#1823, Abcam; 1:1000) with α-mouse secondary (925-32210, LICOR; 1:15000). β-Actin was probed using a 1°-HRP antibody (MA5-15739, Thermo Scientific; 1:1000) and developed using ECL Plus (Thermo Scientific). Detection of blots was performed using LICOR Odyssey LC system and quantified using Image Studio (version 4.0).

TGFβ Luciferase Reporter Assay. ASC were seeded at a density of 10^4 cells/well in a 96-well plate and incubated for 24 hr. Cells were transfected with XFECT transfection reagent

(Clontech) using 1 μg total DNA per sample optimized per the manufacturers' protocol. Briefly, cells were transfected using 500 ng of an SBE-4-Luc plasmid (#16495, Addgene), 500 ng of validated shRNAs (SHC002, Sigma-Aldrich), and 10 ng CMV-LacZ (β-Gal) plasmid for 6 hrs. Media was replaced with fresh 2% FBS DMEM:F12 with either 0, 1, 10, or 75 ppb iAs and cells were allowed to incubate for 48 hrs. Cells were collected in 1X Passive Lysis Buffer (Promega) and luciferase expression was detected using Luciferase Assay System (Promega) and a Glomax 96-well luminometer (Promega).

Statistical Analysis. Unless otherwise described, all assays were run in triplicate with values shown as the mean ± SEM or 95% confidence interval. For pairwise analysis Student's t-test was used, while 1 or 2-way ANOVA were used for comparisons across groups. *P*-values < 0.05 were considered to be statistically significant.

RESULTS

iAs Induced ASC Cytotoxicity. ASC were examined to elucidate the effects of iAs exposure on stromal cell-cell communication. To determine the cytotoxic effects of iAs exposure we exposed naïve primary ASC (P<10) from each donor to concentrations of iAs ranging from 1-100 μM (corresponding to 75-7500 ppb) for 48 hrs. Subsequent cell viability was measured by CCK-8 analysis as described in *Materials and Methods*. IC50 values (Figure 1) demonstrated some biological variability in iAs sensitivity between donors $IC50_{Donor1}$ = 19.3 μM ($CI_{95\%}$=15.5-24.1 μM); $IC50_{Donor2}$ = 12.8 μM ($CI_{95\%}$=10.5-15.7 μM). Based on results from the ASC viability assay and the literature (Ravenscroft 2007) we chose to employ three exposure levels of iAs for subsequent experiments. The exposures chosen were 1, 10, and 75 ppb to appropriately reflect sub-EPA, EPA, and super-EPA levels, respectively (EPA 2001). Additionally, these exposures

represented nonlethal concentrations of iAs that better reflect the environment in which humans are exposed.

Assessment of Prostate Cancer Cell Viability in Co-culture with iAs-Exposed CM. We next examined the effects of ASC CM on PC3 viability. ASC were exposed to either 0 or 75 ppb iAs for one week, after which CM was collected. The one week time point was chosen because previous literature suggests that following chronic exposure, arsenic metabolites reach equilibrium in the urine of exposed individuals (human) in approximately one week (Buchet et al. 1981), reflecting a steady-state exposure. We designed our CM studies to contain 1% CM from ASC relative to cultured PC3 cells reflecting a biologically relevant concentration of MSC localized within the prostate microenvironment (Brennen et al. 2013). Following exposure of 0 or 75 ppb iAs-treated 1% CM, PC3 cell viability was determined at 48 and 96 hrs. Our results suggest CM from ASC exposed to iAs for one week increased prostate cancer cell viability both at 48 and 96 hrs (176 ± 21% $p = 0.022$ and 173 ± 11% $p = 0.003$), respectively (Figure 2, green bars) compared to 2% FBS DMEM:F12 media alone. These results suggest that exposure to iAs may alter ASC in a manner that modifies how the stroma communicates with prostate tumor cells by augmenting tumor cell viability.

Effect of iAs Exposure on Global Cytokine Expression by ASC. Stromal-epithelial communication is known to be an important process in prostate cancer tumorigenesis and is mediated through soluble factors such as cytokines and growth factors (Barron and Rowley 2012). Based on the aforementioned co-culture results which suggest iAs-exposed ASC CM enhance prostate cancer cell viability, we next examined whether a potential mechanism for this observed change could involve disruption of the paracrine cytokine profiles of ASC. Utilizing a cytokine array we measured average cytokine levels between both ASC donors in order to

determine whether iAs mediates changes in their cytokine profiles. Figure 3 displays cytokines that met two major criteria including detectable levels of cytokine in the media, and the presence of concentration-dependent alteration in cytokine levels with exposure. These criteria were used since we were particularly interested in iAs-induced changes and not necessarily in determining the basal cytokine levels. Cytokine expression levels were very similar between donors across the array regarding the increase or decrease in cytokine expression with increasing iAs dose, however the magnitudes differed for some cytokines, suggesting once again some biological variance. When analyzing the mean expression levels of cytokines, we observed upregulation of pro-tumorigenic cytokines (VEGF and IL-8), whereas anti-tumorigenic cytokines (IL-1ra, IP-10, and IFNγ) were downregulated (Figure 3). In addition, a concentration-dependent increase in IL-12 was observed with iAs exposure. These results suggest that iAs exposure may alter a wide range of cell-cell communication that could prime the stromal microenvironment to facilitate tumor progression.

Proteomic Analysis of iAs-Exposed ASC. In order to elucidate potential molecular mechanisms underlying the iAs-induced alteration of stromal cytokines, we performed a proteomic study of ASC from two biological donors. ASC from each donor were exposed to 0 or 75 ppb iAs for one week prior to proteomic analysis. We identified 1,114 unique proteins, of which 440 were upregulated and 170 were downregulated significantly ($p < 0.05$) between 0 and 75 ppb samples in both ASC donors. Quantitative proteomic results were entered into PANTHER Gene Ontology (Mi et al. 2013) to ascertain what global pathways were dysregulated following iAs exposure. PANTHER analysis is summarized in Figure 4. Interestingly, approximately 16% of identified proteins had functions related to cellular processes such as cell movement, cytokinesis, cell cycle, chromosomal segregation, and cell communication. These

processes are interesting future avenues of research for investigating whether they are important stromal-epithelial signaling pathways disrupted during cancer progression following exposure to environmental toxicants. These results suggest that exposure to iAs over a short period of time may promote extensive rewiring of signaling in the stroma composing the tumor microenvironment.

Potential Mechanism of iAs-Induced Perturbations in ASC. Proteomic analysis identified proteins associated with signaling pathways that potentially could underlie the paracrine effect mediated by iAs-treated ASC on prostate cancer viability. IPA was utilized to infer associations between significantly altered protein expression, including HMOX1 and THBS1. Both of these proteins have been associated with the TGFβ signaling pathway (Fitchev et al. 2010; Gueron et al. 2009; Okita et al. 2013). However, at this point the mechanism interconnecting HMOX1 and THBS1 is still incompletely understood.

Proteomic anaylsis showed that the combined average expression (both ASC donors) of HMOX1 was upregulated ~13-fold ($p = 2.8\text{x}10^{-6}$) and THBS1 was downregulated ~3-fold ($p = 1.3\text{x}10^{-6}$). HMOX1, whose expression has been linked to arsenic related oxidative stress response (Reichard et al. 2008), has a recently identified role in prostate tumor progression (Was et al. 2010). While one of the major functions of THBS1 in the prostate is to convert latent TGFβ into its active form (Fitchev et al. 2010), it can also serve as a potential biomarker to distinguish benign prostatic hyperplasia from malignant prostate cancer in epithelia (Shafer et al. 2007), suggesting its role in prostate cancer progression. Our data suggests that altered expression of these proteins may be associated with iAs exposure in ASC.

Focusing on these two candidate genes/proteins, we proceeded to validate the proteomic and IPA analyses by utilizing qRT-PCR and Western blot analysis. Figure 5A shows a

representative Western blot for both proteins normalized to the housekeeping protein, β-Actin. Our results show a concentration-dependent increase in HMOX1 transcript and protein levels normalized with β-Actin for both donors (Figure 5B-C) compared to donor specific unexposed ASC. Additionally, there appears to be an inverse relationship between iAs exposure and THBS1 transcript levels in both donors at a 75 ppb exposure (Figure 5D), whereas protein levels are significantly decreased in both donors at this level of iAs exposure (Figure 5E). Interestingly, Donor 1 had a statistically significant increase in THBS1 mRNA levels at both 1 and 10 ppb unlike Donor 2. Although this is statistically significant, it may not be biologically relevant as it is a relatively modest increase of < 2-fold. Additionally, it may be that Donor 1 is just not as sensitive to iAs exposure as Donor 2, which our cytotoxic data corroborates (Figure 1) and which may explain why discrepancies in mRNA expression are observed at the lower exposures. The protein level data, conversely, appear to correlate better with statistical significance at the higher iAs exposures. These results appear to validate the inferred IPA link between arsenite, HMOX1, THBS1, and the TGFβ pathway, especially at the higher exposure levels. These data suggest a potential pathway which may be responsible for altering paracrine signaling between the stroma and tumor cell populations after exposure to iAs.

TGFβ Signaling in iAs-Exposed ASC. TGFβ signaling is an important signaling pathway in tumorigenesis, acting as both a tumor suppressor and promoter molecule, depending on the microenvironment context (Principe et al. 2014). A decrease in stromal-mediated TGFβ signaling has been linked recently to increased malignancy in prostate cancer (Li et al. 2008). This led us to examine whether iAs exposure was capable of attenuating stromal TGFβ signaling as inferred by IPA. We transfected ASC with a luciferase construct that is a reporter of TGFβ pathway activity since it contains four binding sites for phosphorylated SMAD2/3/4 regulators

(SBE4-Luc, Figure 6A) (Zawel et al. 1998). Unexposed and iAs-exposed ASC were subsequently monitored for luciferase levels following 48 hrs of exposure. Donor 2 was selected for luciferase reporter assay analysis due to its higher transfection efficiency. Results from our luciferase reporter assay suggested that exposure to iAs (1-75 ppb) attenuated TGFβ signaling in ASC, with levels of downregulation ranging from ~43-52% as compared to 0 ppb (unexposed) ASC (Figure 6B). ASC exposed to 75 ppb was the only statistically significantly exposure group when all groups were compared by one-way ANOVA analysis.

DISCUSSION

The ramifications of stromal signaling dysregulation in the context of cancer progression are still being unraveled. In prostate cancer, for instance, the tumor microenvironment has been identified as an important factor associated with cancer progression (Barron and Rowley 2012). To the best of our knowledge, no previous reports have examined the effect of environmental contaminants in stromal cells or the ability of iAs exposure to mediate pro-tumorigenic effects by impacting the stroma.

Attenuation of TGFβ signaling in the stroma has been shown recently to be a critical player in the formation of reactive stroma (Banerjee et al. 2014). However, up until this point there has been little direct evidence for the promotion of an adverse stromal cell reaction by environmental toxicants in an analogous manner. We demonstrate here that the environmental contaminant iAs is capable of altering global signals mediated by stromal cells, potentially through the downregulation of TGFβ signaling. This effect has important implications in that a more reactive stroma might develop following iAs exposure, leading to enhanced prostate cancer progression.

One of the hallmarks of cancer progression is the uncontrolled proliferation of tumor cells. Stromal cells can contribute paracrine signals (growth factors) that can influence strongly the growth of surrounding epithelial cells (Witz 2008). Using a conditioned media co-culture model, we have demonstrated that iAs has the potential to alter the secretory signature of stromal cells by modifying the proteomic and expression profiles of ASC. iAs significantly modified ASC towards a signaling signature that would be consistent with stimulation of prostate cancer cell growth. The main implication of these findings is that environmental toxicants can impact the stromal compartment to augment tumor promotion, especially in the context of inflammation.

To better understand the effects of iAs treated ASC on enhancing prostate tumor cell viability, mediated through paracrine interactions, we investigated a panel of alterations using a cytokine array. ASC are enriched in the prostate microenvironment of prostate cancer patients (Ribeiro et al. 2012c). Ribeiro et al. suggested prostate cancer progression is facilitated by stromal-mediated signaling, which increases hypercellularity and decreases antitumor immunity (Ribeiro et al. 2012a). In line with Ribeiro's evidence, we demonstrated a concentration-dependent relationship in the increase of pro-tumorigenic cytokines (IL-8 and VEGF) and a decrease in anti-tumorigenic cytokines (IL1Ra, IP10, and IFN-γ) following exposure to iAs. Stromal IL-8 expression has been linked to prostate cancer growth and metastatic ability (Thorpe 2013). While substantial evidence has suggested a role for VEGF in prostate cancer progression (Roberts et al. 2013), only recently has its stromal expression been implicated as well (Ding et al. 2013).

Simultaneously, an increased stromal contribution of protumorigenic cytokines and concentration-dependent decreases in antitumorigenic cytokines IL-1Ra, IP-10, and IFN-γ expression were identified. Expression of IL-1Ra (Voronov et al. 2003), IP-10 (Nagpal et al.

2006), and IFN- γ (Hastie et al. 2008) all have been linked to a decrease in prostate cancer severity. Interestingly, the concentration-dependent increase of the proinflammatory cytokine IL-12 may suggest an important role for immune system activation in the tumor microenvironment as IL-12 production has been shown to be important in T-cell maturation (Manetti et al. 1993), meriting further studies *in vivo* in the context of a competent immune system. These results suggest that iAs can modulate changes in the tumor microenvironment by altering cell-cell communication, which in turn is responsible for balancing protumorigenic and antitumorigenic signals.

Additionally, we have described a novel mechanism of action of iAs that may be associated with stromal-epithelial communication, which may involve the HMOX1/THBS1/TGFβ signaling axis. HMOX1 expression previously has been shown as an indicator of arsenic-induced oxidative stress in cells (Reichard et al. 2008). However, a recent review has highlighted a different function for HMOX1 in tumor progression (Was et al. 2010). Activation of HMOX1 as a nuclear receptor has been linked to increased VEGF expression, which may constitute an important link between some of the global cytokine changes detected in our study and HMOX1 expression (Gueron et al. 2009). On the other hand, HMOX1 expression also has been described as inversely related to THBS1 expression (Gueron et al. 2009). THBS1 is an antitumorigenic factor essential for normal prostate angiogenesis whose loss has been linked to tumor progression and enhanced VEGF expression as well (Doll et al. 2001). THBS1 also has been shown to be essential for the conversion of latent TGFβ into its active form which is required to initiate the subsequent signaling cascade (Fitchev et al. 2010). Because TGFβ is a soluble paracrine factor the potential loss of activation by a decrease in stromal THBS1 levels may result in a decrease in stromal derived TGFβ concentration into the microenvironment

niche, potentially explaining the decrease in TGFβ signaling. Disruption of stromal TGFβ signals in prostate cancer already has been identified as an important mediator of prostate cancer progression in the context of bone lesions (Li et al. 2012). This information, combined with the recent report suggesting alterations to TGFβ signaling in stroma as a causative agent of epithelial lesions (Banerjee et al 2014), suggests that the TGFβ pathway must be tightly regulated in stroma in order to maintain epithelial tissue integrity. Interestingly, we have shown that an environmental contaminant is able to induce a similar effect on the stroma as achieved by the genetic ablation of TGFβ receptor, by potentially attenuating TGFβ signaling. Our findings suggest that environmental contaminants may have the potential to create an environment that is suitable for the progression of cancer within the stroma, predisposing the epithelia to tumorigenesis. This mechanism could eventually have important therapeutic implications by targeting the stromal compartment of the tumor microenvironment.

CONCLUSIONS

Our results demonstrate the biochemical complexity associated with iAs carcinogenesis. We demonstrated the capability of iAs to remodel the topology of ASC signaling, potentially modulating a variety of processes such as cell proliferation, angiogenesis, and immune surveillance in the tumor microenvironment. Our findings support the hypothesis that iAs exposure affects not only existing tumors but also is able to impact the environment surrounding epithelial cells, which may increase the predisposition for tumor formation. By better understanding how the stromal microenvironment can be modulated by environmental stressors, we may gain better insight into the etiology of cancer. This could eventually lead to the development of adjuvant strategies aimed at targeting the tumor stroma, which may improve current interventions that suffer from a lack of efficacy.

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FIGURE LEGENDS

Figure 1: ASC Cell Viability After iAs Exposure. Graph represents ASC cell viability after 48 hrs of iAs exposure for two biological ASC donors. Results are displayed as the mean ± SEM (n =3) normalized to percent control (0 ppb) for each individual donor. Dotted line represent the IC50.

Figure 2: iAs-Exposed ASC CM Alters PC3 Cell Viability. 0 or 75 ppb iAs exposed CM was collected from ASC after one week exposure and applied to PC3 cells. Graph represents PC3 cell viability after 48 and 96 hrs compared to control media (2% FBS DMEM:F12 media). Results are displayed as the mean ± SEM (n =3). Pairwise comparison was conducted using Student's t-test with a p-value < 0.05 (*) considered significant.

Figure 3: Concentration Dependent Alteration of Global Cytokine Profile in ASC After iAs Exposure. Results from Bio-Plex Pro™ Human Cytokine 27-plex Assay (Bio-Rad) are displayed in the heat map format (red = upregulation, green = downregulation). The average % change of cytokine levels were determined by pooling ASC Donor 1 and 2 and taking the average expression after one week exposure and comparing iAs exposed values versus 0 ppb exposed ASC. Heat map represent the cytokines that had detectable expression levels and showed a concentration dependent expression profile.

Figure 4: Proteomic Analysis of iAs-Induced Changes in ASC Signaling. Proteins determined to have statistically significant (p < 0.05, two-way ANOVA) differential expression between 0 and 75 ppb iAs after one week of exposure in both donors by proteomic analysis were uploaded into online PANTHER GO Analysis program (http://www.pantherdb.org/).

Figure 5: Validation of IPA Predicted HMOX1/THBS1/TGFβ Signaling Pathway. ASC were exposed to varying concentrations of iAs (0-75 ppb) for one week and mRNA and protein levels were subsequently determined. (A) Representative Western blot (B) HMOX1 mRNA expression (C) HMOX1 protein expression (D) THBS1 mRNA expression (E) THBS1 protein expression. Expression is quantified as mean ± SEM (n =3) compared to donor specific unexposed sample. Statistically significance (*) of $p < 0.05$ was calculated by two-way ANOVA.

Figure 6: iAs Decreases ASC TGFβ Signaling. (A) Schematic representation of SBE4-Luciferase signaling construct. (B) SBE4-Luciferase signaling for ASC Donor 2 exposed to iAs for 48 hrs. Results are displayed as mean ± SEM (n =3). Statistically significance was determined by one-way ANOVA (*p*-value (*) of < 0.05) when comparing 75 ppb to the other three exposure groups (0, 1, 10 ppb).

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Figure 1

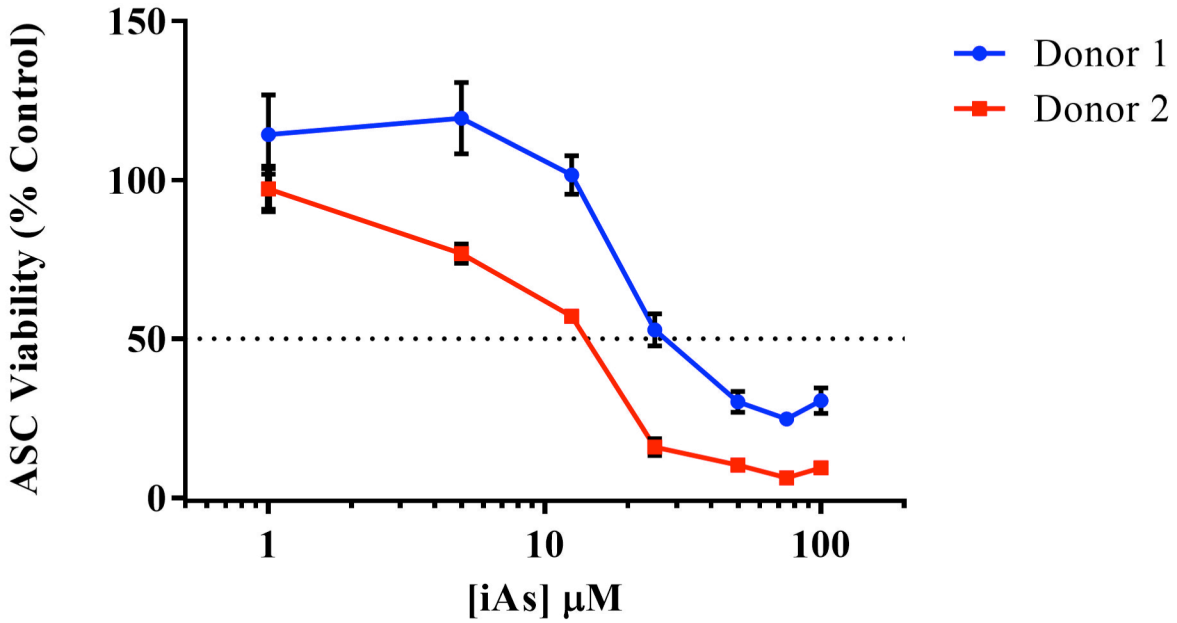

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Figure 2

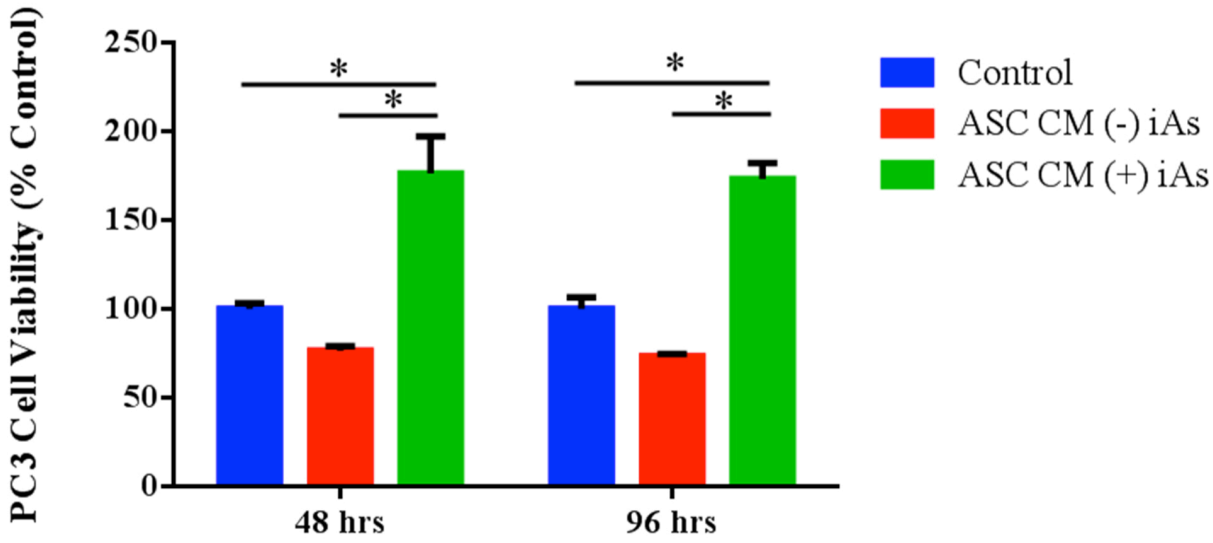

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Figure 3

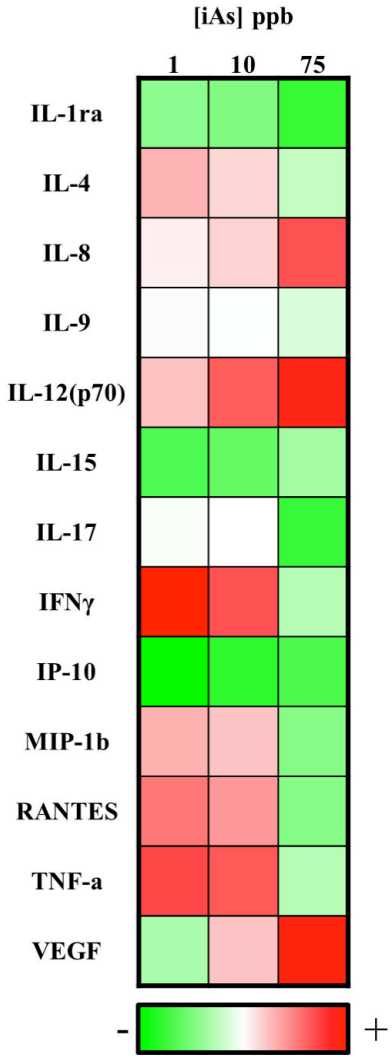

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Figure 4

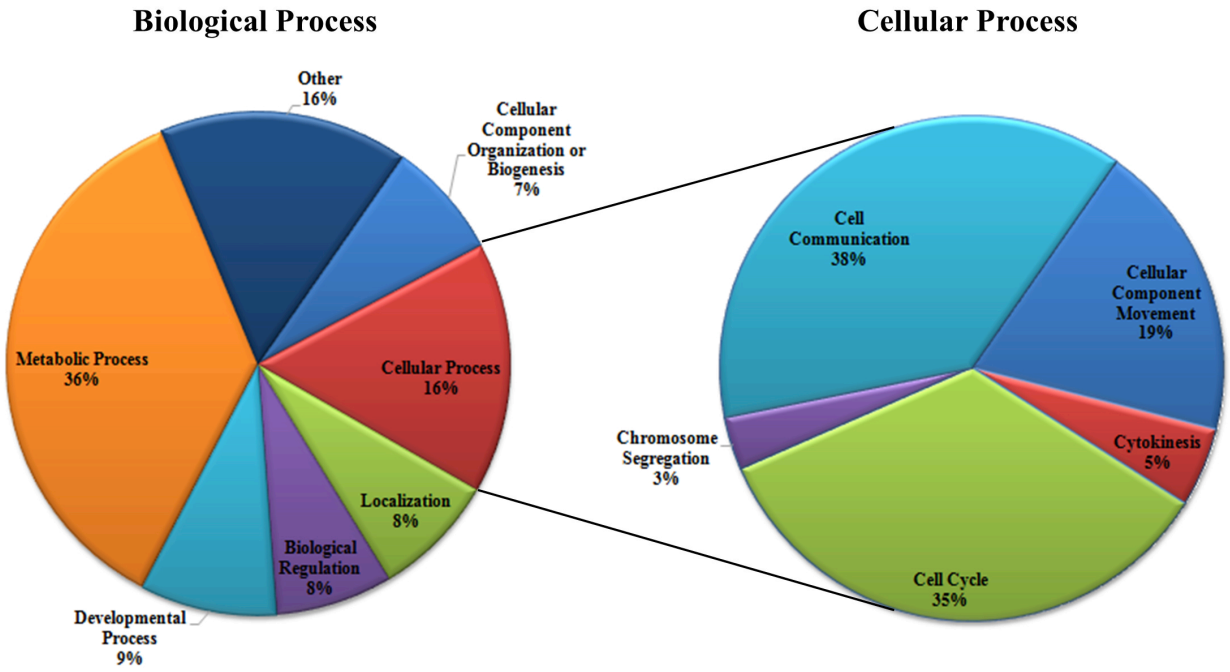

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Figure 5

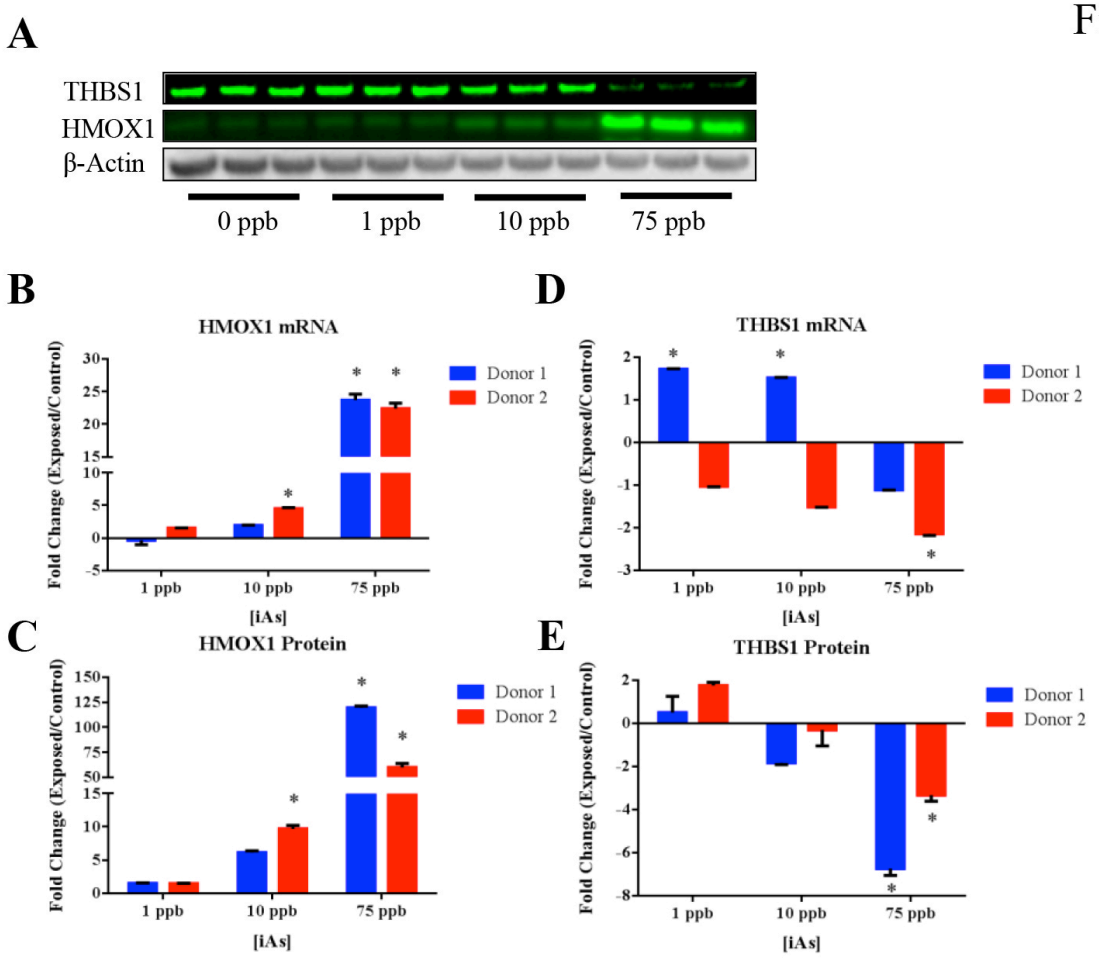

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Figure 6